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Production and Optimization of Alkaline Cellulase from Bacillus Subtilis in Submerged Fermentation

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Abstract: ***Abundant amount of agricultural ,industrial and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes.Cellulose ,a polymer of glucose residues connected by ß-1,4 linkages, being the primary structural material of the plant cell wall, is the most enoromous carbohydrate in the nature.Therefore ,considerable economic interest has been aroused to develop processes for effective treatment and utilization of cellulosic wastes. Cellulase is the enzyme that catalyse the hydrolysis of cellulose into sugar which can be used for food, fuel and value added products. On the other hand , Cellulase are industrially important enzymes with a current annual market value of about 190 million US $.These enzymes have found many industrial applications. Primary among the applications that have been developed for the use of cellulolytic enzymes are those involving textiles, detergent, food, animal feed, bio-fuel, paper and pulp, pharmaceutical and waste management .Cellulases are either intracellular or extracellular. Although a large number of microorganisms can degrade cellulose, only a few produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose. Keeping in view , the demand for new enzymes some microorganisms capable of cellulase production are being isolated and studied.***

Keywords: Cellulose, cellulase enzyme, industrial importance, microorganisms, Bacillus subtilis

1. Introduction

Lignocellulose comprises more than 60 % of plant biomass produced on earth, which includes various agricultural residues (straws, hulls, stems, and stalks), deciduous and coniferous woods, municipal solid wastes, waste from the pulp and paper industry, and herbaceous energy crops, which mainly consists of three components: cellulose, hemicellulose and lignin. The compositions of these materials vary. The major component is cellulose (35–50%), followed by hemicellulose (20–35%) and lignin (10–25%). On worldwide basis, land plants produce about 25 tones of cellulose per person per year. Proteins, oils, and ash make up the remaining fraction of lignocellulosic biomass . The structure of these materials is very complex, and native biomass is generally resistant to an enzymatic hydrolysis.

Nowadays enormous amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Celluloses are regarded as the most important renewable resource\for bioconversion. Many Cellulosic substances were hydrolyzed to simple sugars for making Single Cell Protein, sweeteners etc. It has been become the economic interest to develop aneffective method to hydrolyze the cellulosic biomass. Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters[1] . Increasing knowledge of mode of action of Cellulase; they were used in enzymatic hydrolysis of cellulosic substances [2]. Although a large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro.* Numerous investigations have reported the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement. Fungi are the main cellulase-producing microorganisms, though a few bacteria have also been reported to yield cellulase activity.

Cellulases hold many potential industrial applications. In textile industries, they were used for the “Biopolishing” of fabrics for increasing its softness and brightness. They were also used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. It plays a major role in the conversion of renewable cellulosic biomass into commodity chemicals [3,4]. Lee and Koo [1] showed that cellulase production was the most expensive step during ethanol production from cellulosic biomass, in that it accounted for approximately 40% of the total cost. The chance to obtain cheap ethanol will depend on the successful screening of novel cellulase producing strain. Since industrial bioconversions of lignocelluloses requires multifunctional cellulase with broader substrate utilization as well as the application of enzymes that can work efficiently in a wide range of temperatures and pH conditions used in the bioconversion of cellulosic material to bioethanol. The aim of this study was to isolate and identify new cellulose producing bacteria from soil samples contaminated with decaying lignocellulosic wastes, effluents of paper and pulp industry and cotton industry.

2. Material and Method

2.1 Chemicals and Reagents

Chemicals were obtained from Sigma Chemicals Co. All the chemicals and reagents used for the study were of analytical /microbiological grade and were obtained from commercial vendor.

2.2 Isolation and Screening

Isolation of cellulase **(**predominantely CMCase**)** producing alkalophilic bacteria by enrichment method. Different soil samples contaminated with decaying lignocellulosic wastes, effluents of paper and pulp industry and cotton industry

were collected from various places. One gram of soil was suspended in 25ml sterile deionized water, pH 9 containing 2% cellulose for 48 hrs at 50°C for the enrichment of cellulolytic micro-organisms. For screening, a 100µl aliquot of clear suspension of soil sample was plated onto nutrient-agar carboxymethyl cellulose (CMC) medium, pH 9 containing (g/l):- Peptone 5.0, Beef extract 3.0, Agar 15.0 and CMC 2.5. The colonies found on the plates were spotted onto fresh nutrient agar-CMC plates. After 24 hrs incubation at 50°C, replica plates were made and incubated under the same conditions. Cellulase producing strains were selected by flooding replica plates with 0.5% (w/v) congo red for 15 min. followed by repeated washing with 1M NaCl for zone analysis.

2.3 Identification of Bacteria

Gram staining was performed to check the morphology of the cells. Spore chain morphology was identified by spore staining technique. The isolates were biochemically characterized .Pure culture of the target Bacteria was grown overnight on Nutrient Broth for the isolation of DNA. The DNA was isolated from the bacteria using Cell Lysis method and 16S rDNA was amplified by Thermocycler (PTC – 100 TM Programmable Thermal Controller, USA).The amplified 16S rDNA PCR product was sequenced using automated sequencer. The Sequence Similarity Search was done for the 16S rDNA sequence using online search tool called BLAST(http://www.ncbi.nlm.nih.gov/blast/). The sample 88 was identified using the maximum
aligned sequence through BLAST search[7].

2.4 Production of Cellulase

Cellulase was produced under submerged fermentation in 250 ml erlenmeyer flasks containing 25 ml of basal medium (g/l: peptone, 5.0; yeast extract, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.1) supplemented with 2% wheat bran. The flasks were inoculated with 2% of inoculum (24 hr old) and incubated at 37°C for 48 h under shaking conditions (200 rpm). Crude enzymes were harvested by centrifuging at 10,000g for 20 min at 4°C and the clear supernatant was used as the source of enzymes.

2.5 Assay of Cellulase

1%; carboxymethyl cellulose was used as substrates for assaying the activity of cellulase. The reaction mixture for cellulase assay contained 480 µl of CMC as substrate (prepared in glycine NaOH buffer of pH 9) and 20 µl of enzyme and was incubated at 55°C for 10 min. The reaction was terminated by adding 1.5 ml of 3, 5- dinitrosalicylic acid reagent. Control for the enzyme assay was run simultaneously. The contents were boiled for 15 min and after cooling, the colour developed was read at 540 nm. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar (glucose for cellulase) per minute under the assay conditions.

3. Optimization of Cellulase Production under Submerged Fermentation

The production profile of cellulase was evaluated using different parameters for obtaining the optimum conditions for simultaneous enzymes production. The 18 h old inoculum of size 3% was optimum for maximum cellulase production. Cellulase production was stimulated by Mn^{2+} ions.

4. Methodology

During isolation, cellulase production was carried out in 250 ml conical flask containing 25 ml of basal medium (supplemented with wheat bran). The medium:flask volume ratio was 01:10. Later on, due to the uncompatibility of such a high medium:flask volume ratio at large scale, the optimization was done at the medium:flask volume ratio of 1:5. Under unoptimized conditions (Basal medium composition g/l: peptone, 5.0; yeast extract, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.1; pH 8; supplemented with 2% wheat bran the flasks were inoculated with 2% of 24-h-old inoculum and incubated at 37°C for 48 h under shaking conditions at 200 rpm) with 1:5 medium:flask volume ratio, 6.4 IU/ml of Cellulase was obtained.

The optimization of culture conditions was done through a conventional method ‘one-variable-at-a-time’ strategy. The following parameters were optimized for maximum cellulase production. Incubation period, Inoculum age and size , Media-flask volume ratio, Agitation ,pH ,Temperature, Carbon source and the concentration of selected carbon source, Nitrogen source and the concentration of selected nitrogen source, Metal ions and the concentration of selected metal ion, Effect of various additives.

4.1 Effect of Incubation time

Different incubation times (12, 24,36, 48, 60, 72, 84 hours) were employed to study their effect on the cellulase production. The culture filtrates were collected at respective time interval and assayed.

4.2 Effect of Inoculum age

Different inoculums age (6,12,18, 24,30,36,42, 48, hours) were employed to study their effect on the cellulase production. The culture filtrates were collected and assayed.

4.3 Effect of inoculum size

The cellulase production was observed to be maximum using 3% inoculum. from 0.5% to 3.0% and the culture filtrates were collected and assayed.

4.4 Effect of media-flask volume ratio

Study the effect of media –flask volume (1:2, 1:5,1:10) for maximum cellulase production.

4.5 Effect of agitation rate

The production was carried out at different RPM such as (50,100,150,200,250,300) to study their effect on cellulase production. The culture filtrates were then collected and assayed.

4.6 Effect of Temperature

The production was carried out at different temperatures such as 30, 37, 40, 45, 50,55,60°C to study their effect on cellulase production for 72 hours. The culture filtrates were then collected and assayed.

4.7 Effect of pH

The pH of the production medium was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5,9.0,9.5,10.0,10.5,11.0,11.5,12 with 1N NaOH and 1N HCl. The production was carried out to study their effect on enzyme production.

4.8 Effect of carbon sources

To identify the suitable carbon source for the Cellulase production, the carbon source (CMC) of the production medium was replaced with various carbon sources like Sucrose Mannose, Lactose, Glucose and starch, mannitol ,cellulose ,maltose. Few agro-residues supported the enzymes production. Wheat bran,Wheat husk, rice husk ,sugarcane bagasse, molasses The assay was carried out after 72 hours of incubation.

4.9 Effect of Nitrogen sources

The production of cellulase was optimized by supplementing different nitrogen sources like Beef extract, urea, yeast extract, peptone, pot. Nitrate, ammonium phosphate, ammonium chloride, ammonium sulphate.

4.10Effect of metal ions

Different metal ions showed different effect on cellulase activity. Metal ions like Fe^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+} , **Mg^{2+}, Mn^{2+}, Ca^{2+}** are added to medium to show the effect of metal ions on cellulase production. The culture filtrates were collected and assayed.

4.11Effect of various additives

Various additives used to study the effect of various additives on cellulase production such as Tween 20 ,EDTA Triton X-100, SDS, Glycerol, Oleic acid ,CTAB. The culture filtrates were collected and assayed.

5. Result and Discussion

Since most of the natural wastes were degraded by the native microbes present in it, the present study deals with the analyzing the microbes present in the soil samples contaminated with decaying lignocellulosic wastes, effluents of paper and pulp industry and cotton industry for their ability of producing Cellulase. Bacterial samples were isolated from the soil of which, best strains which produces better zone producing strain(fig 1) was chosen and preceded for further studies [14]. Cellulase producing Bacteria were found commonly in all environments which enables them to degrade the cellulose found prevalent in waste materials.

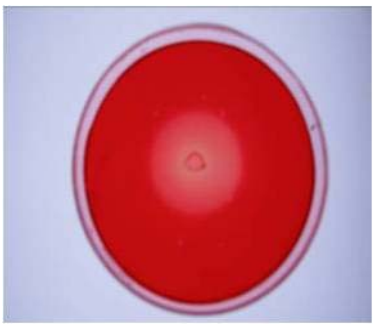

Figure 1: Screening of cellulase producing bacteria

The better zone producing strain was assigned name as sample 88 . The DNA from the strain 88 was isolated and the 16S rDNA was amplified and sequenced. The BLAST analysis of the strain using its 16S rDNA sequence data showed that strain 88 had highest homology (100 %) with *Bacillus subtilis*. When compared to morphological and biochemical characterization methods(table 1), 16S rDNA analysis is found to be the novel and accurate method for identifying unknown species. 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level.

Table showing results of bacterial isolation for cellulase production

Source of bacteria	*Bacterial Isolate No.*	*Zone on CMC-Agar media*	*Diameter of zone (mm.)*	*Enzyme Activity (IU/ml)*
Degrading wood soil, KUK, Sample-A	1	+	7±1	1.5±0.7
	2	+	10±1	2.8±0.08
	3	+	11±1	3.1±0.2
	4	-	-	
	5	-	-	
	6	+	12±1	3.4±0.07
Degrading wood soil, KUK, Sample-B	7	-	-	
	8	-	-	
	9	+	14±1	3.1±0.7
	10	+	15±1	2.5±0.5
Degrading wheat field sample, Narwana	11	-	-	
	12	+	8±1	3.7±0.5
	13	-	-	
	14	-	-	
Manure soil sample, Narwana	15	+	15±1	3.8±0.6
	16	-	-	
	17	-	-	
	18	-	-	
	19	+	7±1	2.1±0.1
Degrading wood soil, YNR	20	+	6±1	1.9±0.6
	21	-	-	
	22	-	-	

	23	-	-	
Degrading saw dust sample,YNR, Sample-A	24	+	12±1	2.8±0.5
	25	+	10±1	2.6±0.2
	26	-	-	
	27	-	-	
	28	+	15±1	3.1±0.6
	29	+	8±1	2.8±0.2
Degrading saw dust sample,YNR, Sample-B	30	+	13±1	3.7±0.35
	31	-	-	
	32	-	-	
	33	+	9±1	3.1±0.75
	34	+	8±1	3.4±0.5
Degrading paddy soil sample,Narwana	35	-	-	
	36	-	-	
	37	-	-	
	38	-	-	
Degrading cotton soil sample,Narwana	39	+	16±1	3.6±0.9
	40	+	12±1	3.2±0.6
	41	+	20±1	4.0±0.64
	42	-	-	
	43	-	-	
Cotton industry sample A, Hisar	44	+	15±1	4.5±0.22
	45	-	-	
	46	+	22±1	4.7±0.05
	47	-	-	
	48	+	23±1	5.3±0.7
Cotton industry sample B, Hisar	49	+	18±1	4.7±0.06
	50	+	19±1	5.1±0.06
	51	-	-	
	52	+	13±1	4.3±
	53	-	-	
	54	+	12±1	3.8±1
Degrading sugarcane field sample, YNR	55	-	-	
	56	-	-	
	57	+	09±1	2.7±0.34
	58	-	-	
	59	+	07±1	2.6±0.5
Sugar mill sample, YNR	60	+	08±1	2.2±0.7
	61	-	-	
	62	-	-	
	63	-	-	
	64	+	05±1	2.2±0.5
Kraft industry, KKR, sample A	65	+	23±1	5.9±0.5
	66	+	21±1	5.6±0.3
	67	+	21±1	5.2±0.3
	68	-	-	
	69	-	-	
	70	+	18±1	4.8±0.12
Kraft industry, KKR, sample B	71	+	18±1	4.1±0.5
	72	+	24±1	5.4±0.6
	73	-	-	
	74	+	17±1	4.3±0.8
Paper industry, YNR, Sample A	75	+	17±1	4.6±0.5
	76	+	24±1	5.1±1
	77	+	16±1	3.9±0.66
	78	+	21±1	5.8±32
	79	+	19±1	4.1±0.2
Paper industry, YNR, Sample B	80	-	-	
	81	-	-	
	82	-	-	
	83	+	24±1	5.4±0.5
Cotton industry, Zirakpur	84	-	-	
	85	+	14±1	3.8±0.5
	86	-	-	
	87	+	13±1	3.7±0.2
	88	**+**	**26±1**	**6.4±0.5**
	89	+	16±1	4.2±0.1

Abbreviations used :- KKR- kurukshetra, YNR- yamunanagar, KUK-kurukshetra university. Results presented are the mean of three independent experiments with standard error values. a = under unoptimized conditions, Nil = activity not detected

Bacterial isolate 88 was selected for further studies.

Morphological Tests

Colony Morphology	
Configuration	Circular
Margin	Undulate
Elevation	Flat
Surface	Smooth
Pigment	White
Opacity	Opaque
Gram Reaction	+VE
Cell Shape	RODS
Arrangement	Scattered
Spore (S)	+VE

Physiological Tests

Temp Range	10-50
PH RANGE	6-10
GROWTH ON NaCl	2-6%
Groth Under Anaerobic	+

Biochemical Tests

GROTH ON Macconkey	+, Non Lactose Fermentor
Indole Test	-
Methyl Red Test	+
Voges Proskauer Test	+
Citrate Utilization	+
H_2s Production	-
Gas Production From Glucose	-
Casein Hydrolysis	+
Gelatin Hydrolysis	+
Starch Hydrolysis	+
Nitrate Reduction	+
Catalase Test	+
Oxidase Test	-
Esculin Hydrolysis	+
Arginine Dihydrolase	-
Tween 20 Hydrolysis	+
Tween 40 Hydrolysis	+
Tween 60 Hydrolysis	+
Tween 80 Hydrolysis	-
Acid Production From Maltose	+

Acid Production From Glucose	+
Acid Production From Fructose	+
Acid Production From Mannose	+
Acid Production From Mannitol	+
Acid Production From Sorbitol	-

5.1 Molecular characterization of the Isolate 88

The Bacterial isolated in the present study was labeled as **sample 88** was found to be Bacillus subtilis subsp. inaquosorum ***strain*** KCTC 13429(T) (GenBank Accession Number: AMXN01000021) based on nucleotide homology and phylogenetic analysis. It was identified by IMTECH Chandigarh India and has been given National Centre of Biotechnology Information (NCBI) Accession no AMXN01000021 **.**

5.2 Optimisation parameters

As the environmental parameters are essential for the production of Cellulase, they were optimized by shaken flask fermentation method[19] . There is a gradual increase in production occurred from 12th hour (Fig. 2) and maximum production was occurred at 24 hours with the enzyme activity of 9.23±0.20 IU/ml. The Incubation time depends on the nutrients present in the medium and the cultural conditions of the organism[20].

5.3 Time profile of cellulase production

The maximum cellulolytic activity was achieved after 24 h of incubation. (fig 2)

Incubation time (h)	Enzyme Activity (IU/ml)
12	4.36±0.21
24	**9.23±0.20**
36	7.4±0.11
48	6.36±0.29
60	6.2±0.20
72	5.3±0.11
84	3.7±0.20

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

The main parameters like temperature, pH are very essential parameters of the Cellulase production. To optimize the optimum temperature for the better Cellulase production, productions were made in various temperatures. The higher cellulase activity was found as **10.8±0.32** IU/ml at 37°C and at 40°C is **10.13±0.29** IU/ml for the Cellulase production (Fig. 3). The temperature requirement of the organism is based on the nature of organisms.

5.3.1 Effect of incubation temperature on cellulase production

Optimum incubation temperature for enzymes production was found to be 37 ^{0}C .(fig 3)

Incubation temperature (˚C)	Enzyme Activity (IU/ml)
30	10.2±0.80
37	**10.8±0.32**
40	**10.13±0.29**
45	8.02±0.40
50	5.3±0.31
55	1.86±0.37
60	0.36±0.18

Data presented are the average obtained from there independent experiments with standard error (±SE) values.
As the pH is found to be also impotent environmental parameter, varying pH were analyzed on Cellulase production. Maximum production of the enzyme (**10.86±0.41** IU/ml) was obtained at the pH 8.0 (Fig, 4). The pH of the selected organism was closely related to the optimum pH values of most of the *Bacillus* spp.[21].

5.3.2 Effect of pH of the medium cellulase production

pH of the medium is one of the important parameter during enzyme production. Optimum cellulase production was obtained at pH 8. (fig 4)

pH of production media	Enzyme Activity (IU/ml)
6	7.8±0.46
6.5	8.4±0.55
7	9.86±0.46
7.5	**10.43±0.23**
8	**10.86±0.41**
8.5	10.26±0.64
9	8.16±0.27
9.5	8.09±0.40
10	5.36±0.28
10.5	2.42±0.26
11	2.36±0.21
11.5	1.39±0.11
12	1.15±0.21

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.3 Effect of inoculum age on cellulase production

Maximum cellulase activity was attained with 18-h-old inoculums.(fig 5)

Inoculum age (h)	Enzyme Activity (IU/ml)
6	2.73±0.27
12	5.53±0.24
18	**8.43±0.31**
24	**9.53±0.26**
30	8.8±0.21
36	6.13±0.14
42	4.43±0.34
48	1.8±0.17

Data presented are the average obtained from there independent experiments with standard error (±SE) values**.**

5.3.4 Effect of inoculum size on cellulase production

The cellulase production was observed to be maximum using 3% inoculum. Cellulase level increased when the inoculum size increased from 0.5% to 3.0% and decreased with further increase in inoculum size. (fig 6)

Inoculum size (%)	*Enzyme Activity (IU/ml)*
0.5	2.63±0.26
1	5.26±0.32
1.5	5.63±0.40
2	6.8±0.30
2.5	**9.1±0.32**
3	**11.5±0.32**
3.5	9.43±0.53
4	7.43±0.63
4.5	4.4±0.44

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.5 Effect of media-flask volume ratio on cellulase production

Both the enzymes gave maximum activity at media-flask volume ratio 1:10, this is very high ratio not possible to be attained at industrial scale; therefore 1:5 ratio was used for optimization. (fig 7)

Media:flask volume ratio	**Cellulase Activity (IU/ml)**
1:2	6.2±0.46
1:5	10.03±0.37
1:10	13.1±0.61

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.6 Effect of agitation rate on cellulase production

Cellulase production varied considerably with RPM during fermentation. Optimum enzymes yield was obtained with 250 rpm. (fig 8)

Agitation rate (rpm)	**Enzyme Activity (IU/ml)**
50	2.63±0.24
100	4.8±0.37
150	9.0±0.23
200	9.56±0.52
250	**11.4±0.37**
300	10.7±0.40

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.7 Effect of various carbon sources on cellulase production

Among all selected pure carbohydrates (like lactose, maltose, sucrose, and fructose) fructose improved the enzyme production, showing that the enzyme is inducible with these easily metabolizable sugars, while only few agro-residues supported the enzymes production. Wheat bran induced cellulase activity more than other agro-wastes used. Wheat husk, rice husk ,sugarcane bagasse, molasses gave poor cellulase titers. Therefore, further combination of wheat bran and fructose was tested to get the optimum cellulase production .

Carbon Sources	**Enzyme activity (IU/ml)**
Pure carbohydrate (1%)	
Lactose	7.63±0.4
Maltose	5.74±0.72
Mannitol	6.41±0.38
Sucrose	4.22±0.45
Fructose	**10.61±0.41**
Glucose	6.45±0.20
Cellulose	5.63±0.31
Starch	2.33±0.51
Agricultural waste (2%)	
Wheat bran	**11.4±0.47**
Wheat husk	3.26±0.60
Rice husk	3.73±0.86
Rice bran	6.13±0.54
Sugarcane baggase	4.76±0.37
Molasses	3.66±0.29
Orange peel	2.43±0.65
Mousami peel	1.76±0.51
Saw dust	1.43±0.23
Banana peel	7.76±0.29

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.8 Effect of concentration of optimized carbon source (wheat bran and fructose) on cellulase production

In order to determine the best amount of wheat bran and fructose for cellulase production, different concentrations (0.5-5%) of wheat bran and (0.1-1%) fructose were tested. The results showed that highest cellulase (19.6 IU/ml), was obtained with the combination of 3% wheat bran (WB) and 0.25% fructose. Concentration of agro-industrial waste higher than 3% increased the medium viscosity to great extent resulting in reduced oxygen transfer and significant reduction in enzyme titers.

Fructose (%)	*WB*	*WB*	*WB*	*WB*	*WB*
	Enzyme avtivity (IU/ml)				
0.1	7.51±0.	7.54±0.	9.31±0.	10.52±0	10.07±0
0.25	6.81±0.	9.62±0.	**12.6±0.**	10.24±0	11.62±0
0.5	8.32±0.	8.75±0.	11.72±0	9.37±0.	9.56±0.
1	7.46±0.	9.86±0.	11.63±0	9.71±0.	9.38±0.

Data presented are the average obtained from there independent experiments with standard error (±SE) values**.**

5.3.9 Effect of various nitrogen sources on cellulase production

The impact of various organic and inorganic nitrogen sources was evaluated on cellulase production. Among the nitrogen sources tested the highest cellulase activity was achieved by yeast extract and peptone. The results showed that the organic nitrogen sources were stimulating the enzyme production. But, the inorganic nitrogen sources were less efficient and even use of many of them resulted in significant decline in enzyme titers. Supplementation of the production medium with a combination of peptone, and yeast extract made appreciable difference in enzyme

production when compared to peptone and yeast extract alone.

Nitrogen	**Enzyme**
Organic cources	
Peptone	**11.33±0.21**
Yeast extract	**11.72±0.66**
Beef extract	8.03±0.23
Casein	9.06±0.31
Urea	8.13±0.27
Inorganic sources	
Potassium	5.1±0.15
Ammonium	1.2±0.47
Ammonium	2.96±0.48
Ammonium	6.03±0.49

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.10 Effect of different concentration of the selected nitrogen source on cellulase production

A gradual increase in enzyme production was observed with increase in the concentration of peptone and yeast extract upto 0.5%.

Peptone	**YE (0.1%)**	**YE (0.25%)**	**YE (0.5%)**	**YE (1%)**
	Enzyme avtivity (IU/ml)			
0.1	5.92±0.33	6.9±0.55	5.5±0.84	09.3±0.2
0.25	8.31±0.22	8.9±0.2	12.6±0.73	10.5±0.5
0.5	12.4±0.2	12.9±0.41	12.4±0.35	11.1±0.35
1	10.2±0.2	10.2±0.64	13.4±0.4	13.6±0.

Data presented are the average obtained from there independent experiments with standard error (±SE) values.

5.3.11 Effect of metal ions on cellulase production

Different metal ions showed different effect on cellulase activity. Where on one side **Mg^{2+}, Mn^{2+}, Ca^{2+}** enhanced enzyme activity. Some of metal ions like Fe^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+} inhibited activity sharply.

Metal ion (5mM)	*Enzyme activity (IU/ml)*
Fe^{2+}	7.23±0.24
Hg^{2+}	2.4±0.37
Cd^{2+}	1.5±0.20
Zn^{2+}	3.8±0.55
Co^{2+}	5.8±0.32
Cu^{2+}	4.32±0.24
Mg^{2+}	10.1±0.15
Mn^{2+}	11.31±0.66
Ca^{2+}	9.8±0.41
Ni^{2+}	8.2±0.34
K^{+}	8.7±0.20

Data presented are the average obtained from there independent experiments with standard error (±SE) values

5.3.12 Effect of concentration of Mn^{2+} ions on cellulase production

Concentration of Mn^{2+} (%)	*Enzyme activity (IU/ml)*
0.02	5.6±0.2
0.03	5.5±0.4
0.04	5.7±0.2
0.05	9.9±0.2
0.06	**10.3±0.34**
0.07	**11.4±0.5**
0.08	12.8±0.2
0.09	11.8±0.18
1.00	11.3±0.1

Data presented are the average obtained from there independent experiments with standard error (±SE) values

5.3.13 Effect of NaCl on cellulase production

No major effect of NaCl was observed on cellulase activity.

NaCl (%)	*Enzyme activity (IU/ml)*
0.25	10.2±0.34
0.5	10.1±0.6
1	11±0.2
2	10.2±0.16

Data presented are the average obtained from there independent experiments with standard error (±SE) value..

5.3.14 Effect of various additives on cellulase production

Among various additives used Tween 20 and EDTA showed the negligible increase in cellulase titer where as all others (Triton X-100, SDS, Glycerol, Oleic acid) strongly inhibited cellulase activities.

Additive (0.2%)	**Enzyme activity (IU/ml)**
CTAB	2.6±0.55
EDTA	**12.3±0.32**
Triton X100	4.3±0.21
SDS	6.4±0.2
Glycerol	7.6±0.3
Oleic acid	7.02±0.55
Oil of Olive	9.1±0.64
Tween 20	11.45±0.38

Data presented are the average obtained from there independent experiments with standard error (±SE) values. The commonly used animal feeds like sugarcane bagasse, sugar beet pulp/husk, orange bagasse, oil cakes, apple pomace, grape juice, grape seed, coffee husk, wheat bran, cereals, straw, leaves, corncobs were disposed in environments [27]. They were degraded by bacteria and fungi. So, those waste materials can be used as substrates for the cellulase production [28].

6. Conclusion

In the present study, *maximum* production of cellulase was obtained from *Bacillus subtilis* Selected bacterial stain under unoptimized conditions gave 6.4 IU/ml. The organism will be further identified by molecular method like 16srDNA analysis to confirm their novelty. Optimum cellulase activity was found after 24 h of incubation. The 18 h old inoculum of size 3% was optimum for maximum cellulase

production.Enzymes gave maximum activity at media-flask volume ratio 1:10, this is very high ratio not possible to be attained at industrial scale; therefore 1:5 ratio was used for optimization.

Optimum enzymes production was found at pH 8 and incubation temperature 37°C.Cellulase production was evaluated in presence of several carbon and nitrogen sources. Out of various carbon sources, combination of wheat bran (3%) and fructose (0.25%) enhanced cellulase production.Organic nitrogen source (peptone and yeast extract) enhanced the cellulase production at 0.5% concentration each. Cellulase activities were stimulated by Mn^{2+} ions. Under optimized conditions, cellulase gave 12.8±0.66 IU/ml.

7. Future Scope

The application of cellulase produced from *Bacillus subtilis* is in biostoning of denim fabric.

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